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(57) Abstract

An *in vivo* modification process is described. The process affects the mannose-to-galactose ratio of either an organism (or part thereof) capable of producing a mannose/galactose containing compound or of a mannose/galactose containing compound thereof. The *in vivo* modification process comprises expressing a nucleotide sequence that has an effect on: (a) the mannose-to-galactose ratio of mannose and galactose components of a mannose/galactose containing compound; and/or (b) the mannose-to-galactose ratio of mannose and galactose precursors for a mannose/galactose containing compound; and wherein the nucleotide sequence is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme.

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IN VIVO MODIFICATION OF GALACTOMANNANS IN GUAR BY EXPRESSION OF UDP–GALACTOSE EPIMERASE ANTISENSE RNA

The present invention relates to a modification process.

In particular, the present invention relates to an *in vivo* modification process.

Galactomannans are a heterogenous group of cell wall polysaccharides consisting of a β-1-4 linked mannan backbone with varying numbers of α-1-6 linked galactose side chains.

The galactomannans of most significant industrial use are obtained from the endosperms of the legumes guar (*Cyamopsis tetragonolobus*) and locust bean (*Ceratonia siliqua*). These galactomannans differ in their galactose content, with guar having a galactose to mannose ratio of approximately 1:1.6, whereas the ratio for locust bean gum (LBG) is approximately 1:3.4.

The differences in galactose content have significant effects on the functional properties of guar gum and LBG. Both galactomannans form highly viscous solutions at low concentrations (1-2%) but LBG has the additional property of being able to form firm gels with other polysaccharides such as xanthan, carrageenan and agarose. LBG is extensively used by the food industry in dairy products (notably ice cream), salad dressings, sauces, low calorie products and pet foods. However, the use of LBG is restricted by the high price and irregular supply.

Therefore, large scale production of galactomannans with improved functional properties, such as a result of an increased mannose to galactose ratio (such as that similar to LBG), is desirable.

Due to the generic chemical similarity between guar gum and LBG and the much lower price on guar gum, it has been attempted *in vitro* to convert guar gum into a galactomannan with LBG-like properties and with a chemical composition similar to LBG.

An example of such an *in vitro* treatment includes the use of α-galactosidase. In this regard, see McCleary *et al* 1983 and EP-A-0255153.

By using α-galactosidase purified from guar seeds, guar gum with galactose contents of 10-34% was obtained (Bulpin *et al* 1990). Analysis of the gelation behaviour of the modified guar gum showed that a preparation with a galactose content of 24% formed mixed gels with carrageenan displaying similar rheological properties as LBG. In comparison, the galactose content of untreated guar gum was 38% and 23% for LBG.

However, from an industrial point of view *in vitro* degalactosylation of guar gum is associated with a number of problems.

First, huge amounts of α-galactosidase have to be prepared as about 40% of the galactose in the guar gum has to be removed.

Second, during incubation it is very important that no hydrolysis of the mannan backbone occurs necessitating the use of highly purified α-galactosidase preparations devoid of any trace of mannanase activity. A procedure for heterologous production of the α-galactosidase from guar seed has been published (Overbeeke *et al* 1986). However, the produced α-galactosidases from the tested species were purified before investigating the action on guar gum suggesting that the mannanase problem remains to be solved.

Third, the yield of galactomannan is reduced because a 40% reduction in galactose content corresponds to approximately 15% less modified guar gum. The released galactose may be undesirable in the final product and may have to be removed.

Fourth, there is a considerable risk for depolymerisation of the galactomannan during incubation with α-galactosidase.

Also, there is a risk for contaminating microorganisms to colonise the reaction mixture releasing endo-β-mannanases.

Finally, water has to be removed from the reaction mixture. In addition to the cost of this process, it will also result in concentration of the buffer that may be used for obtaining optimal reaction conditions.

These examples demonstrate that the present methods for the modification of guar gum are associated with problems, some of which are associated with considerable costs.

There is therefore a need to have an improved method for the modification of guar gum.

In this regard, we have already proposed the modification of a mannose/galactose containing compound (such as guar gum) wherein the modification occurs *in vivo* in plants, such as guar plants, and by use of recombinant DNA techniques. In particular, we refer to PCT/EP96/05581 which was filed 2 December 1996 (the contents of which are incorporated herein by reference).

In its broadest sense, PCT/EP96/05581 relates to *in vivo* modification of a mannose/galactose containing compound - such as guar gum - in an organism (or part thereof) capable of synthesising that compound by a method that is not native to that organism - such as by a method that makes use of recombinant DNA techniques. The modification may occur in relation to any one or more of the precursors of the compound (e.g. mannose and/or galactose) or in relation to the compound itself (i.e. modification of the mannose and/or galactose units of a compound comprising same).

In particular, PCT/EP96/05581 relates to an *in vivo* modification process that affects, preferably increases, the mannose-to-galactose ratio of either an organism (or part thereof) capable of producing a mannose/galactose containing compound or of a mannose/galactose containing compound thereof. This *in vivo* modification process is not a naturally occurring process.

Thus, with the *in vivo* process of PCT/EP96/05581, it is possible to alter the internal *in vivo* ratio of mannose to galactose within an organism and/or the ratio of mannose to galactose of a mannose/galactose compound thereof.

One of the requirements for the production of *in vivo* modified guar gum is the availability of a method for the introduction of suitable genes into guar. This has been accomplished to a limited extent by Jørsboe and Okkels (1994) who transferred a selectable and screenable gene used for the development of the transformation method. These authors did not report on transformation with a gene to affect the mannose to galactose ratio. This is an important point as, from a biotechnological point of view, the major obstacle for the production of *in vivo* modified guar gum is the lack of knowledge of galactomannan biosynthesis. Up until now, no genes or gene products which control the biosynthesis of guar gum *in vivo* have been isolated and characterised. However, in PCT/EP96/05581 we determined some of the genes or gene products which control the biosynthesis of guar gum *in vivo* - thus enabling us to modify guar gum *in vivo*.

We now provide a new process for the modification of a mannose/galactose containing compound (such as guar gum).

According to a first aspect of the present invention we provide an *in vivo* modification process that affects the mannose-to-galactose ratio of either an organism (or part thereof) capable of producing a mannose/galactose containing compound or of a mannose/galactose containing compound thereof, wherein the *in vivo* modification process comprises expressing a nucleotide sequence that has an effect on: (a) the mannose-to-galactose ratio of mannose and galactose components of a mannose/galactose containing compound; and/or (b) the mannose-to-galactose ratio of mannose and galactose precursors for a mannose/galactose containing compound; and wherein the nucleotide sequence is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme.

According to a second aspect of the present invention we provide the use of a nucleotide sequence to affect *in vivo* the mannose-to-galactose ratio of either an organism (or part thereof) capable of producing a mannose/galactose containing compound or of a mannose/galactose containing compound thereof, wherein the nucleotide sequence is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme, and wherein the nucleotide sequence has an effect on: (a) the mannose-to-galactose ratio of mannose and galactose components of a mannose/galactose containing compound; and/or (b) the mannose-to-galactose ratio of mannose and galactose precursors for a mannose/galactose containing compound.

In a preferred embodiment, the nucleotide sequence is antisense to at least a part of the coding sequence for a UDP-galactose epimerase enzyme.

The term "mannose/galactose containing compound" means a compound comprising at least one mannose group and at least one galactose group.

It is preferred that the mannose/galactose containing compound is galactomannan.

It is more preferred that the mannose/galactose containing compound is guar gum.

It is more preferred that the organism capable of producing a mannose/galactose containing compound is a guar plant and the mannose/galactose containing compound thereof is galactomannan. However, other galactomannan producing plants are encompassed such as fenugreek and lucerne. Plants that are considered not to produce appropriate quantities of galactomannan belong to the family *Solanacea* and the species *Nicotiana tabacum*.

The term "organism (or part thereof) capable of producing a mannose/galactose containing compound" also includes any suitable organism - in particular a plant - capable of producing a mannose/galactose containing compound, such that the internal *in vivo* ratio of mannose to galactose of that organism is altered. The term also includes any part of an

organism that is capable of producing a mannose/galactose containing compound, such that the ratio of mannose to galactose of that part is altered. The term also includes a part when within an organism or in a live culture medium. Preferably, the part is when within an organism *per se*. An example of a part is seed.

The term "mannose and galactose precursors" includes mannose *per se* or derivatives thereof and/or galactose *per se* or derivatives thereof as precursors for the biosynthesis of a mannose/galactose containing compound, preferably galactomannan. In addition, the term includes precursors for mannose *per se* or derivatives thereof and/or galactose *per se* or derivatives thereof which in turn are used as precursors for the biosynthesis of a mannose/galactose containing compound, preferably galactomannan. Preferably, the term means mannose *per se* or derivatives thereof (such as mannose-6-phosphate or GDP-mannose) and/or galactose *per se* or derivatives thereof as precursors for the biosynthesis of galactomannan, preferably guar galactomannan.

Preferably the *in vivo* mannose-to-galactose ratio of the organism (or part thereof) or mannose/galactose containing compound thereof is higher than that of the guar plant or the galactomannan thereof.

More preferably the *in vivo* mannose-to-galactose ratio of the organism (or part thereof) or mannose/galactose containing compound thereof is substantially similar to that of the locust bean or the galactomannan thereof.

Preferably the organism (or part thereof) or mannose/galactose containing compound thereof is a guar plant or the gum thereof.

The present invention also covers a mannose/galactose containing compound when prepared by the process of the present invention. This mannose/galactose containing compound will be referred to as a mannose/galactose containing compound according to the present invention.

In addition, the present invention also covers a foodstuff comprising a mannose/galactose containing compound according to the present invention.

In addition, the present invention also covers a composition - such as a foodstuff - comprising a mannose/galactose containing compound according to the present invention admixed with another polysaccharide. Preferably that other saccharide is any one or more of xanthan, carrageenan and agarose.

In addition, the present invention covers methods for preparing compositions or foodstuffs according to the present invention comprising mixing the mannose/galactose containing compound according to the present invention with another suitable ingredient.

The broad aspects of the present invention can be achieved by the use of a nucleotide sequence that is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme. UDP-galactose epimerase enzyme converts UDP-glucose to UDP-galactose which is incorporated into the galactomannan. With this strategy, expression of antisense UDP-galactose epimerase reduces the epimerase activity and thereby increases the *in vivo* mannose-to-galactose ratio of mannose and galactose precursors for the biosynthesis of a mannose containing compound, such as galactomannan.

A preferred aspect of the present invention relates to a construct comprising or expressing the nucleotide sequence of the present invention.

Another preferred aspect of the present invention relates to a vector comprising or expressing the construct or nucleotide sequence of the present invention.

Another preferred aspect of the present invention relates to a plasmid comprising or expressing the vector, construct or nucleotide sequence of the present invention.

Another preferred aspect of the present invention relates to a transgenic organism comprising or expressing the plasmid, vector, construct or nucleotide sequence of the present invention.

Other preferred aspects of the present invention include methods of expressing or allowing expression or transforming any one of the nucleotide sequence, the construct, the plasmid, the vector, the cell, the tissue, the organ or the organism, as well as the products thereof.

Further preferred aspects of the present invention include uses of the antisense nucleotide sequence according to the present invention for preparing or treating foodstuffs, including animal feed.

One of the key advantages of the present invention is that by using the anti-sense sequence it is possible to increase the mannose-to-galactose ratio of organisms or mannose containing compounds thereof, in particular *in vivo* modified guar gum.

The antisense nucleotide sequence may be used *in vivo* in combination with one or more other nucleotide sequences, which nucleotide sequences are preferably prepared by use of recombinant DNA techniques.

Likewise the nucleotide sequence of the present invention may be used in combination with one or more gene products to further affect the mannose-to-galactose ratio, which gene products are preferably prepared by use of recombinant DNA techniques. Here, the gene products can be any one or more of peptides, polypeptides, proteins, enzymes and RNA. Preferably, at least one of the gene products is an enzyme that is expressed by a nucleotide sequence that is not a natural nucleotide sequence. Here, the term "a natural nucleotide sequence" means an entire nucleotide sequence that is in its natural environment and when operatively linked to an entire promoter with which it is naturally associated, which promoter is also in its natural environment.

By way of example, in some instances it is preferred that the antisense nucleotide sequence according to the present invention is used in conjunction with one or more genes encoding enzymes that are required for the biosynthesis of GDP-mannose - namely the enzyme phosphomannose isomerase (PMI) and/or the enzyme phosphomannose mutase and/or the enzyme GDP-mannose pyrophosphorylase - and/or with one or more genes encoding an α-galactosidase.

In a more preferred instance the nucleotide sequence according to the present invention is used in conjunction with a gene encoding the enzyme phosphomannose isomerase (PMI) and/or with a gene encoding an α-galactosidase. A preferred PMI and α-galactosidase are disclosed and discussed in PCT/EP96/05581 (the contents of which are incorporated herein by reference).

The present invention also encompasses the use of variants, homologues or fragments of the nucleotide sequence according to the present invention. Here the terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence is similar to a nucleotide sequence that is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme and wherein the resultant nucleotide sequence is capable of affecting the mannose-to-galactose ratio as defined above. These terms are synonymous with allelic variations of the sequences.

The UDP-galactose epimerase enzyme - the gene sequence of which the nucleotide sequence of the present invention is antisense to - may be any suitable UDP-galactose epimerase enzyme. Preferably the UDP-galactose epimerase enzyme - the gene sequence of which the nucleotide sequence of the present invention is antisense to - is an endogenous UDP-galactose epimerase enzyme or an enzyme having a sequence similar thereto (such as at least 85% sequence similarity, preferably at least 90% sequence similarity, more preferably at least 95% sequence similarity, more preferably at least 98% sequence similarity).

In accordance with a preferred embodiment of the present invention the UDP-galactose epimerase enzyme - the gene sequence of which the nucleotide sequence of the present invention is antisense to - may be or may comprise the amino acid sequence as presented in SEQ ID No. 1 or SEQ ID No. 2, or a variant, homologue or fragment thereof.

In accordance with a more preferred embodiment of the present invention the UDP-galactose epimerase enzyme - the gene sequence of which the nucleotide sequence of the present invention is antisense to - may be or may comprise the amino acid sequence as presented in SEQ ID No. 1 or SEQ ID No. 2.

In addition, and according to a further preferred embodiment of the present invention the nucleotide sequence of the present invention is antisense to the UDP-galactose epimerase enzyme coding sequence that is presented in SEQ ID No. 1 or SEQ ID No. 2, or a variant, homologue or fragment thereof.

According to a further preferred embodiment of the present invention the nucleotide sequence of the present invention is antisense to the UDP-galactose epimerase enzyme coding sequence that is presented in SEQ ID No. 1 or SEQ ID No. 2.

As indicated above, the terms "variant", "homologue" or "fragment" in relation to the amino acid sequence for the preferred UDP-galactose epimerase enzyme *vis-a-vis* the antisense nucleotide sequence of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant enzyme has UDP-galactose epimerase activity, preferably having at least the same activity of the enzyme comprising the sequence shown as SEQ ID No. 1 or SEQ ID No. 2. In particular, the term "homologue" covers homology with respect to structure and/or function. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to an enzyme comprising the sequence shown as SEQ ID No. 1 or SEQ ID No. 2. More preferably there is at least 95%, more preferably at least 98%,

homology to an enzyme comprising the sequence shown as SEQ ID No. 1 or SEQ ID No. 2.

The terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence coding for the UDP-galactose epimerase enzyme *vis-a-vis* the antisense nucleotide sequence of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for or is capable of coding for an enzyme having UDP-galactose epimerase activity, preferably having at least the same activity of the enzyme comprising the sequence shown as SEQ ID No. 1 or SEQ ID No. 2. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence codes for or is capable of coding for an enzyme having UDP-galactose epimerase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to a sequence comprising the sequence shown as SEQ ID No. 1 or SEQ ID No. 2. More preferably there is at least 95%, more preferably at least 98%, homology to a sequence that comprises the sequence shown as SEQ ID No. 1 or SEQ ID No. 2.

The terms "variant", "homologue" or "fragment" in relation to the antisense nucleotide sequence according to the present invention - namely a sequence that is antisense to that coding for the UDP-galactose epimerase enzyme - include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence is antisense to a sequence that codes for or is capable of coding for an enzyme having UDP-galactose epimerase activity, preferably having at least the same activity of the enzyme comprising the sequence shown as SEQ ID No. 1 or SEQ ID No. 2. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence is antisense to a sequence that codes for or is capable of coding for an enzyme having UDP-galactose epimerase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to a sequence comprising a sequence that is antisense to the sequence shown as SEQ ID No. 1 or SEQ ID No. 2. More preferably there is at least 95%, more

preferably at least 98%, homology to a sequence comprising a sequence that is antisense to the sequence shown as SEQ ID No. 1 or SEQ ID No. 2.

The transgenic organism of the present invention includes an organism comprising any one or more of the nucleotide sequences according to the present invention, constructs according to the present invention, vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention, or the products thereof, including combinations thereof. For example the transgenic organism can also comprise any one or more of the nucleotide sequences of the present invention under the control of one or more heterologous promoters.

In a highly preferred embodiment, the transgenic organism (or part thereof) does not comprise the combination of a promoter and the nucleotide sequence according to the present invention, wherein both the promoter and the nucleotide sequence are native to that organism (or part thereof) and are in their natural environment.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Mond theory of gene expression. The promoter could additionally include one or more features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoters may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable other sequences include the *Sh1*-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements.

Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97).

Thus, in one aspect, the nucleotide sequence according to the present invention is under the control of a promoter that allows expression of the nucleotide sequence. In this aspect, the promoter may be a cell or tissue specific promoter. If, for example, the organism is a plant then the promoter can be one that affects expression of the nucleotide sequence in any one or more of seed, stem, sprout, root and leaf tissues.

By way of example, the promoter for the nucleotide sequence of the present invention can be the α-Amy 1 promoter (otherwise known as the Amy 1 promoter, the Amy 637 promoter or the α-Amy 637 promoter) as described in PCT/EP95/02195.

Alternatively, the promoter for the nucleotide sequence of the present invention can be the α-Amy 3 promoter (otherwise known as the Amy 3 promoter, the Amy 351 promoter or the α-Amy 351 promoter) as described in PCT/EP95/02196. With the Amy 351 promoter it is possible to inactivate a part of it so that the partially inactivated promoter expresses the nucleotide sequence in a more specific manner such as in just one specific tissue type or organ. The term "inactivated" means partial inactivation in the sense that the expression pattern of the promoter is modified but wherein the partially inactivated promoter still functions as a promoter. However, as mentioned above, the modified promoter is capable of expressing the nucleotide sequence in at least one (but not all) specific tissue of the original promoter. Examples of other partial inactivation of a promoter sequence (and not just necessarily that of the Amy 351 promoter) include altering the folding pattern of the promoter sequence, or binding species to parts of the nucleotide sequence, so that a part of the nucleotide sequence is not recognised by, for example, a specific RNA polymerase. Another, and preferable, way of partially inactivating the Amy 351 promoter is to truncate it to form fragments thereof. Another way would be to mutate at least a part of the sequence so that the RNA polymerase can not bind to that part or another part.

Another modification is to mutate the binding sites for regulatory proteins for example the CreA protein known from filamentous fungi to exert carbon catabolite repression, and thus abolish the catabolite repression of the native promoter.

The present invention therefore concerns affecting mannose-to-galactose ratios by the use of recombinant DNA techniques.

General teachings of recombinant DNA techniques may be found in Sambrook, J., Fritsch, E.F., Maniatis T. (Editors) Molecular Cloning. A laboratory manual. Second edition. Cold Spring Harbour Laboratory Press. New York 1989.

The present invention also concerns affecting mannose-to-galactose ratios of or within a plant - such as by preparing a transgenic plant. Even though the enzyme and the nucleotide sequence of the present invention are not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to prepare transgenic plants according to the present invention. An adaption of some of these background teachings is now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries the nucleotide sequence or construct according to the present invention and which is capable of introducing the nucleotide sequence or construct into the genome of an organism, such as a plant. The vector system may comprise one vector, but it can comprise two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector

system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists,* eds.: D.S. Ingrams and J.P. Helgeson, 203-208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

The nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the terminal sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appears to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct.

Furthermore, the vector system is preferably an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant the nucleotide sequence or construct of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful

microorganism is *E. coli*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli*, it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the nucleotide sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the nucleotide sequence or construct of the invention, which DNA is subsequently transferred into the plant cell to be modified.

As reported in CA-A-2006454, a large amount of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the transformed cells. The vectors contain for example pBR 322, pUC series, M13 mp series, pACYC 184 etc.

In this way, the nucleotide or construct of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of analysis there is generally used sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

After each introduction method of the construct or nucleotide sequence according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema. in: The Binary Plant

Vector System Offset-drukkerij Kanters B.B., Alblasserdam, 1985, Chapter V; Fraley, et al., Crit. Rev. Plant Sci., 4:1-46; and An et al., EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists,* eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant.

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the nucleotide sequence of the present invention, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium.* The inoculated plant or plant part is then grown on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc.

Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Further teachings on plant transformation may be found in EP-A-0449375.

Even further useful teachings on the transformation of plants can be found in Danish patent application No. 940662 (filed 10 June 1994) and/or United Kingdom patent application No. 9702592.8 (filed 7 February 1997).

Reference may even be made to Spngstad *et al* (1995 Plant Cell Tissue Organ Culture **40** pp 1-15) as these authors present a general overview on transgenic plant construction.

By way of example, transgenic guar plants according to the present invention can be achieved by transformation of guar cotyledonary explants by the method according to Joersbo and Okkels (PCT/DK95/00221) using *Agrobacterium tumefaciens* LBA4404.

The following samples were deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 23 May 1997:

1. *E.coli* DH5αpGEPI42. The deposit number is NCIMB 40881. NCIMB 40881 contains clone GEPI42 - which comprises SEQ ID No. 1.

2. *E.coli* DH5αpGEPI48. The deposit number is NCIMB 40882. NCIMB 40882 contains clone GEPI48 - which comprises SEQ ID No. 2.

Thus, according to a preferred embodiment the nucleotide sequence of the present invention is antisense to the UDP-galactose epimerase enzyme coding sequence that is obtainable from deposit number NCIMB 40881 or NCIMB 40882, or is a variant, homologue or fragment thereof.

According to a more preferred embodiment the nucleotide sequence of the present invention is antisense to the UDP-galactose epimerase enzyme coding sequence that is obtainable from deposit number NCIMB 40881 or NCIMB 40882.

The present invention will now be described only by way of examples.

Cloning and partial characterization of UDP-galactose epimerase genes from guar (*Cyamopsis tetragonoloba*)

Biochemical studies on UDP-galactose 4-epimerase (EC 5.1.3.2.) in guar have shown that even though fairly high activity of this enzyme is found, the amount of UDP-galactose epimerase protein was very small preventing preparative purification for amino acid analysis. Therefore, the cloning strategy of choice was by functional complementation in an *galE- E. coli* mutant.

cDNA library

A cDNA expression library representing mRNA from immature guar seeds was constructed in the plasmid pcDNAII (Invitrogen Corporation) and transformed into the *E. coli* strain Top10F'. The quality of the cDNA library was controlled by purification of plasmids from a number of separate Top10F' colonies, picked at random. Restriction enzyme analysis revealed that all examined plasmids were recombinant.

UDP-galactose epimerase deficient *E. coli* strain

The *E. coli* strain PL-2 is not able to metabolise galactose due to a defective *galE-* gene while the two other genes of the gal-operon, *galK* and *galT*, are intact (Buttin, J Mol Biol, 7, 164-182 (1963); Wu and Kalckar, Proc Nat Acad Sci, USA 55, 622-629 (1966). Thus, insertion of an active UDP-galactose epimerase gene in PL-2 would allow this strain to grow on galactose.

Transformation of PL-2 and selection

PL-2 cells were made competent by the method of Hanahan (Techniques for transformation of *E. coli*. IRL Press, Oxford (ISBN 0-947946-18-17), 109-135 (1985). A titer of 5 x 10^6 transformed cells/µg library plasmid was obtained.

The selection medium was essentially a minimal medium added galactose, consisting of M9 salts (Maniatis *et al*, Molecular cloning, a laboratory manual, Cold Spring Harbor, New York (ISBN 0-87969-136-0) (1982) and added 0.05 g/l threonine, 0.05 g/l leucine, 0.05 g/l methionine, 1.0 g/l of thiamin-HC1, 50 mg/l ampicillin, 0.8 g/l fructose, 0.9 g/l agarose and 6 or 8 g/l galactose. The media are hereafter called M9-ES6 (containing 6 g/l galactose) or M9-ES8 (containing 8 g/l galactose).

Competent PL-2 cells were transformed with the guar cDNA library and cells were plated onto the selective substrate M9-ES6 or M9-ES8. After two days at 37°C, colonies appeared (approx 0.1% of the total number of transformants). A total of 48 colonies were selected.

UDP-galactose epimerase assay on selected colonies

In order to establish whether the acquired ability to grow on galactose was due to the presence of UDP-galactose activity, all selected colonies were tested for UDP-galactose epimerase activity using crude extracts produced by sonication and subsequent clarification by centrifugation. The UDP-galactose epimerase assays were performed essentially according to Dey (Phytochem, 23, 729-732 (1984)). The UDP-galactose epimerase activity in extracts of PL-2 was zero (negative control) while significant levels of activity was found in DH5a (positive control), as expected. Transformed PL-2 colonies displaying high levels of UDP-galactose epimerase activity were chosen for further analysis.

Retransformation with plasmid DNA from colonies 42 and 48

Plasmids from colony 42 and colony 48 were purified and retransformed into competent PL-2 cells and plated on M9-ES6 or M9-ES8. In both retransformation experiments, a large number of colonies appeared after two days at 37°C. About ten independent colonies from each were analysed for UDP-galactose epimerase activity and all extracts contained similar high levels of UDP-galactose activity as found in the original colonies 42 and 48. This experiment demonstrates that the UDP-galactose epimerase activity detected in the PL-2 derived colonies 42 and 48 is encoded by the cDNA inserts.

DNA sequencing analysis of the inserts in colonies 42 and 48

Partial nucleotide sequences of the UDP-galactose-4-epimerase containing clones, pGEPI42 and pGEPI48, were determined using a Termo sequenase fluorescent cycle sequencing kit (Amersham) and an ALF DNA sequencer (Pharmacia).

Sequence ID No. 1 and No. 2 show partial nucleotide sequences of the inserts in colonies 42 and 48, respectively, along with the deduced amino acid sequences.

Antisense Experiments

Antisense nucleotide sequences to the above-mentioned clones of interest are prepared and are used to transform guar gum by following the above-mentioned techniques.

Resultant analysis reveals that it is possible to increase the mannose-to-galactose ratio of guar gum by the insertion of one or more of those antisense sequences. Thus, the present invention is based on the surprising finding that it is possible to increase the mannose-to-galactose ratio of guar gum by the insertion of a nucleotide sequence that is an antisense nucleotide sequence.

Other modifications of the present invention will be apparent to those skilled in the art.

REFERENCES

Bojsen *et al* (1993) PCT patent application WO 94/20627

Bulpin P V, Gidley M J, Jeffcoat R, Underwood D R. Development of a biotechnological process for the modification of galactomannan polymers with plant α-galactosidase. Carbohydr. Polymers 1990, 12:155.

Edwards M, Bulpin P V, Dea I C M, Reid J S G. Biosynthesis of legume-seed galactomannan *in vitro*. Planta. 1989, 178:41.

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McCleary B V, Critchley P, Bulpin B V. Processing of polysaccharides. European patent application, 1983, EP-A-0 121 960.

Maniatis T, Fritsch E F, Sambrook J (1982). Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York. ISBN: 0-87969-136-0.

Overbeeke N, Fellinger A J, Hughes S G. Production of guar alpha-galactosidase by hosts transformed by recombinant DNA methods. European patent application, 1986, EP-A-0 255 153.

Reid J S G, Edwards M E. Galactomannans and other cell wall storage polysaccharides in seeds. In: food polysaccharides and their application. Ed: A M Stephen, Marcel Dekker, 1995, pp 155-186.

```
******************************************
* TRANSLATION OF A NUCLEIC ACID SEQUENCE *
******************************************

Done on DNA sequence GEPI42.

Total number of bases is: 381.

        10         20         30         40         50         60
         |          |          |          |          |          |
GAATTCCTGAAATCTGAAGTGTGAAGAAGAATAATAATAAGGAACAGTGAGTGGGATTTG

        70         80         90        100        110        120
         |          |          |          |          |          |
AAGGGAAAGAAGAAGAAGAAGAAGATGGTGTCGTCGAGGATGGCGTCAGGGGAAACAATT
                         M  V  S  S  R  M  A  S  G  E  T  I

       130        140        150        160        170        180
         |          |          |          |          |          |
CTGGTAACTGGAGGAGCTGGATTCATCGGATCTCACACGGTGGTTCAGCTTCTGAAGCAA
L  V  T  G  G  A  G  F  I  G  S  H  T  V  V  Q  L  L  K  Q

       190        200        210        220        230        240
         |          |          |          |          |          |
GGGTTTCACGTATCCATCATCGACAATCTCTACAACTCCGTCATCGACGCCGTCCATAGG
G  F  H  V  S  I  I  D  N  L  Y  N  S  V  I  D  A  V  H  R

       250        260        270        280        290        300
         |          |          |          |          |          |
GTTCGCCTTTTGGTGGGTCCACTCCTCTCCAGCAACCTCCATTTCCATCACGGCGACCTC
V  R  L  L  V  G  P  L  L  S  S  N  L  H  F  H  H  G  D  L

       310        320        330        340        350        360
         |          |          |          |          |          |
CGCAACATCCATGACCTCGACATCCTCTTCTCTAAAACCAAATTTGATGCCGTGATCCAA
R  N  I  H  D  L  D  I  L  F  S  K  T  K  F  D  A  V  I  Q

       370        380
         |          |
CTTGCGGGCCCCAAAGGTGTG
L  A  G  P  K  G  V
```

SEQ ID NO. 1

```
****************************************
* TRANSLATION OF A NUCLEIC ACID SEQUENCE *
****************************************

Done on DNA sequence GEPI48.

Total number of bases is: 351.

        10        20        30        40        50        60
         |         |         |         |         |         |
GAATTCCTTCAAAGCTATCCATACCGCTTACGCATTCATTCACTCCACCTTCTCTTTCTC

        70        80        90       100       110       120
         |         |         |         |         |         |
TCTCAGCTCCCTTCAATTATGTCATCCCAAACGGTTCTCGTCACCGGCGGAGCCGGTTAC
                    M  S  S  Q  T  V  L  V  T  G  G  A  G  Y

       130       140       150       160       170       180
         |         |         |         |         |         |
ATCGGCAGCCACACCGTCCTTCAGCTTCTCCTCGGTGGTTTCAAGGCCGTTGTCGTTGAC
 I  G  S  H  T  V  L  Q  L  L  L  G  G  F  K  A  V  V  V  D

       190       200       210       220       230       240
         |         |         |         |         |         |
AACCTCGATAATTCTTCCGAGACCGCCATCCACAGAGTCAAGGAACTCGCCGGTAAATTC
 N  L  D  N  S  S  E  T  A  I  H  R  V  K  E  L  A  G  K  F

       250       260       270       280       290       300
         |         |         |         |         |         |
GCCGGTAATCTCTCCTTTCACAAGTTAGACCTTCGGGACAGAGATGCGCTGGAAAAAATT
 A  G  N  L  S  F  H  K  L  D  L  R  D  R  D  A  L  E  K  I

       310       320       330       340       350
         |         |         |         |         |
TTTTCTTCCACAAAGTTTGATTCTGTCATACATTTTGCTGGACTGAAAGCA
 F  S  S  T  K  F  D  S  V  I  H  F  A  G  L  K  A
```

SEQ ID NO. 2

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 18, line 12-21

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St Machar Drive
Aberdeen
AB2 1RY
United Kingdom

Date of deposit	Accession Number
23 May 1997	NCIMB 40881

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 18, line 12-21

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Date of deposit	Accession Number
23 May 1997	NCIMB 40882

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*

This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

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E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
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CLAIMS

1. An *in vivo* modification process that affects the mannose-to-galactose ratio of either an organism (or part thereof) capable of producing a mannose/galactose containing compound or of a mannose/galactose containing compound thereof, wherein the *in vivo* modification process comprises expressing a nucleotide sequence that has an effect on:

(a) the mannose-to-galactose ratio of mannose and galactose components of a mannose/galactose containing compound; and/or

(b) the mannose-to-galactose ratio of mannose and galactose precursors for a mannose/galactose containing compound;

wherein the nucleotide sequence is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme.

2. Use of a nucleotide sequence to affect *in vivo* the mannose-to-galactose ratio of either an organism (or part thereof) capable of producing a mannose/galactose containing compound or of a mannose/galactose containing compound thereof, wherein the nucleotide sequence is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme, and wherein the nucleotide sequence has an effect on:

(a) the mannose-to-galactose ratio of mannose and galactose components of a mannose/galactose containing compound; and/or

(b) the mannose-to-galactose ratio of mannose and galactose precursors for a mannose/galactose containing compound.

3. The invention according to claim 1 or claim 2 wherein the mannose/galactose containing compound is galactomannan.

4. The invention according to any one of claims 1 to 3 wherein the organism capable of producing a mannose/galactose containing compound is a guar plant.

5. The invention according to any one of claims 1 to 4 wherein the *in vivo* mannose-to-galactose ratio of the organism (or part thereof) or mannose/galactose containing compound thereof is higher than that of the guar plant or the galactomannan thereof.

6. The invention according to any one of claims 1 to 5 wherein the *in vivo* mannose-to-galactose ratio of the organism (or part thereof) or mannose/galactose containing compound thereof is substantially similar to that of the locust bean or the galactomannan thereof.

7. A construct comprising a nucleotide sequence that is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme.

8. A vector comprising a nucleotide sequence that is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme.

9. A plasmid comprising a nucleotide sequence that is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme.

10. A transgenic organism (or part thereof) comprising a nucleotide sequence that is antisense to at least a part of the gene for a UDP-galactose epimerase enzyme.

11. A transgenic organism (or part thereof) according to claim 10 wherein the organism is a guar plant.

12. The invention according to any of one of the preceding claims wherein the nucleotide sequence is antisense to at least a part of the coding sequence for a UDP-galactose epimerase enzyme.

13. A mannose/galactose containing compound when prepared by the process of claim 1 or any claim dependent thereon.

14. A foodstuff comprising a mannose/galactose containing compound according to claim 13.

15. A composition comprising a mannose/galactose containing compound according to claim 13 admixed with another polysaccharide.

16. A composition comprising a mannose/galactose containing compound according to claim 13 admixed with any one or more of xanthan, carrageenan and agarose.

17. A method for preparing a composition or a foodstuff comprising mixing the mannose/galactose containing compound according to claim 13 with another suitable ingredient.

18. A process substantially as described herein.

INTERNATIONAL SEARCH REPORT

Inter ..onal Application No
PCT/IB 98/00891

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/61 C12N15/11 C12N15/82 A01H5/00 C12P19/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BULPIN P V ET AL: "DEVELOPMENT OF A BIOTECHNOLOGICAL PROCESS FOR THE MODIFICATION OF GALACTOMANNAN POLYMERS WITH PLANT -GALACTOSIDASE" CARBOHYDRATE POLYMERS, vol. 12, no. 2, 1 January 1990, pages 155-168, XP000083942 cited in the application see the whole document, especially the abstract, and page 165, last paragraph, which continues on page 166. ---	13-18

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 1998	13/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL - EMPLN Entry PS31544, Acc.No. U31544, 3 February 1996 SLOCUM, R.D. ET AL.: "Pisum sativum UDP-galactose-4-epimerase (galE) mRNA, complete cds." XP002076761 see the whole document ---	7-9
X	DATABASE EMBL - EMPLN Entry OSAD430Q, Acc.No. D21120, 17 November 1993 UCHIMIYA, H.: "Rice mRNA for UDP-galactose 4-epimerase, partial sequence." XP002076762 see the whole document ---	7-9
X	DÖRMANN, P. ET AL.: "Functional expression of Uridine 5'diphospho-glucose 4-epimerase (EC 5.1.3.2) from Arabidopsis thaliana in Saccharomyces cerevisiae and Escherichia coli." ARCHIEVES IN BIOCHEMISTRY AND BIOPHYSICS, vol. 327, no. 1, 1 March 1996, XP002076759 see the whole document ---	7-10
A	EDWARDS ET AL: "Biosynthesis of legume-seed galactomannans in vitro" PLANTA, no. 178, 1989, page 41 41 XP002076760 see page 46, left-hand column, last line - right-hand column, paragraph 1 ---	
A	WO 90 12876 A (DANSKE SPRITFABRIKKER) 1 November 1990 cited in the application see the whole document ---	
P,X	WO 97 20937 A (DANISCO ;JOERSBOE MORTEN (DK); BRUNSTEDT JANNE (DK); PETERSEN STEE) 12 June 1997 cited in the application see the whole document ---	10-14
P,X	DÖRMANN, P. ET AL.: "The role of UDP-glucose epimerase in carbohydrate metabolism of Arabidopsis." PLANT JOURNAL, vol. 13, no. 5, March 1998, pages 641-52, XP002082772 see the whole document -----	1-3, 5-10,12

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 98/00891

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9012876	A	01-11-1990	AU	638248	B	24-06-1993
			AU	5531890	A	16-11-1990
			CA	2053230	A	25-10-1990
			DD	298949	A	19-03-1992
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			DE	69008172	T	20-10-1994
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			ES	2053189	T	16-07-1994
			IE	63067	B	22-03-1995
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			LV	10727	B	20-02-1996
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WO 9720937	A	12-06-1997	AU	1193997	A	27-06-1997
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